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Global Levels of Histone Modifications Predict Prognosis in Different Cancers

David B. Seligson,* Steve Horvath,†‡ Matthew A. McBrian,§ Vei Mah,* Hong Yu,* Sheila Tze,* Qun Wang,* David Chia,* Lee Goodglick,* and Siavash K. Kurdistani*§¶

From the Departments of Pathology and Laboratory Medicine, Human Genetics,† Biostatistics,‡ in the School of Public Health, and Biological Chemistry,§ and the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research,¶ David Geffen School of Medicine, University of California, Los Angeles, California*

Cancer cells exhibit alterations in histone modification patterns at individual genes and globally at the level of single nuclei in individual cells. We demonstrated previously that lower global/cellular levels of histone H3 lysine 4 dimethylation (H3K4me2) and H3K18 acetylation (ac) predict a higher risk of prostate cancer recurrence. Here we show that the cellular levels of both H3K4me2 and H3K18ac also predict clinical outcome in both lung and kidney cancer patients, with lower levels predicting significantly poorer survival probabilities in both cancer groups. We also show that lower cellular levels of H3K9me2, a modification associated with both gene activity and repression, is also prognostic of poorer outcome for individuals with either prostate or kidney cancers. The predictive power of these histone modifications was independent of tissue-specific clinicopathological variables, the proliferation marker Ki-67, or a p53 tumor suppressor mutation. Chromatin immunoprecipitation experiments indicated that the lower cellular levels of histone modifications in more aggressive cancer cell lines correlated with lower levels of modifications at DNA repetitive elements but not with gene promoters across the genome. Our results suggest that lower global levels of histone modifications are predictive of a more aggressive cancer phenotype, revealing a surprising commonality in prognostic epigenetic patterns of adenocarcinomas of different tissue origins. ***(Am J Pathol 2009, 174:1619–1628; DOI: 10.2353/ajpath.2009.080874)***

Cancer is a disease of genetic and epigenetic alterations. Epigenetics include the interrelated processes of DNA methylation and histone modifications, aberrations of which occur commonly in human cancer.[1–3] In the case of histone modifications, these aberrations may occur locally at gene promoters by inappropriate targeting of histone-modifying enzymes, leading to improper expression or repression of individual genes that play important roles in tumorigenesis. For instance, the E2F transcription factor recruits the tumor suppressor retinoblastoma protein to its target genes. Retinoblastoma protein in turn recruits HDAC1, which leads to transcriptional silencing of genes with important roles in tumor biology such as cyclin E.[4,5] Aberrant modification of histones associated with DNA repetitive sequences has also been reported, which include lower levels of H4K16ac and H4K20me3 in hematological malignancies and colorectal adenocarcinomas.[6] Furthermore, when examined at a global level by immunostaining of primary tumor tissues, individual tumor nuclei show variable levels of histone modifications, generating an additional layer of epigenetic heterogeneity at the cellular level.[7] Thus, tumor cells may harbor aberrant patterns of histone modifications at individual promoters, repetitive elements, and globally at the level of single nuclei.

In cancer patients, clinical outcome prediction is based generally on tumor burden and degree of spread with additional information provided by histological type and patient demographics. However, cancer patients

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Address reprint requests to Siavash K. Kurdistani, M.D., David Geffen School of Medicine at UCLA, P.O. Box 951737, Los Angeles, CA 90095-1737. E-mail: skurdistani@mednet.ucla.edu.

with similar tumor characteristics still show heterogeneity in the course and outcome of disease. Therefore, accurate subclassification of patients with similar clinical outcomes is required for development of targeted therapies and personalization of patient care.[8] In this regard, molecular biomarkers have been useful in distinguishing subtypes of cancer patients with distinct clinical outcomes, thereby expanding our prognostic capabilities. Among the various biomarkers, expression analysis of genes, individually or especially in groups as molecular fingerprints,[9] has been used widely to identify disease subtypes with differences in outcome in multiple cancers such as lymphomas[10] and breast cancers.[11–13] Similar to gene expression, DNA methylation of specific genes have also been used as biomarkers, especially in predicting response to treatments.[14] For instance, in gliomas, methylation status of MGMT (O^6-methylguanine-DNA methyltransferase) promoter region correlates with response or resistance to alkylating agents.[15]

We showed previously that heterogeneity in cellular (ie, global or bulk) levels of histone modifications can be detected by immunohistochemistry (IHC) at the level of whole nuclei of cancer cells in tissue specimens.[7] In prostate cancer tissue from an individual patient, malignant cells exhibit dissimilar levels of histone modifications. The extent of dissimilarity in the levels of histone modifications—quantified as percent cell staining—differs between patients. These differences generate epigenetic patterns that, in the case of prostate cancer, predict risk of tumor recurrence after removal of the primary tumor. Of the five modifications that we examined in prostate cancer, H3K4me2 and H3K18ac proved to be the most informative of prognosis. The cellular patterns of these two modifications were sufficient to distinguish two groups of patients with distinct clinical outcomes, whom otherwise were not distinguishable by standard clinicopathological variables.[7] In general, patients with low cellular levels of H3K4me2 and H3K18ac (ie, decreased percent cell staining) had poorer prognosis with significantly increased risk of tumor recurrence compared with patients with higher levels of the two modifications. These findings demonstrated a novel link between cellular epigenetic heterogeneity and clinical behavior in cancer patients.

Considering that histones and their modifications are present ubiquitously, our results in prostate cancer raised the possibility that histone modification patterns may serve as markers of prognosis in other cancer types. Furthermore, the prognostic utility of histone modifications may not be limited to the modifications examined so far. Other histone modifications may provide improved or complimentary prognostic capability. With respect to the gene expression prognosticators, expression of one or more genes can be predictive of clinical outcome, but in most cases the identity of prognosticator genes is different in different cancers. Extending this logic to epigenetics, one would expect that different histone modifications predict prognosis in different cancers. However, we provide evidence here that the lower cellular levels of the same two histone modifications that were most informative in prostate cancer, H3K4me2 and H3K18ac, distinguish patients with decreased survival probabilities in other adenocarcinomas (ie, cancer of glandular epithelium), namely, cancers of lung and kidney. We did not examine the levels of the other three modifications from our original study.[7] However, we show that the cellular levels of another histone modification, H3K9me2, which is associated with gene activity and repression, is by itself a strong predictor of clinical outcome, with lower levels predicting poor outcomes in prostate and kidney cancers. Consistent with primary tissues, we show that prostate cancer cell lines also exhibit different cellular levels of histone modifications. These global differences in cancer cell lines are correlated with changes in histone modification levels at repetitive DNA elements and less so with promoter regions. Our findings suggest that the cellular levels of histone modifications may be general predictors of clinical outcome in adenocarcinomas of different tissue origins; and that global loss of histone modifications may be linked to a more aggressive cancer phenotype.

Materials and Methods

Sample Collection and Tissue Microarrays (TMAs)

After UCLA Institutional Review Board approval, formalin-fixed. paraffin-embedded specimens of benign and tumor tissues from human lung, kidney, and prostate were obtained from the Department of Pathology from surgical cases occurring between 1984 and 2002. Sample collection was blinded to clinical data, which were obtained after TMA construction. At least three tumor tissue core biopsies 0.6 mm in diameter were taken from selected morphologically representative regions of each paraffin-embedded sample and arrayed as described previously.[16] Tumor staging for all tissue types was performed according to the American Joint Committee on Cancer and the International Union Against Cancer tumor-node-metastasis (TNM) classification of malignant tumors. T stage was determined from surgical pathology, N and M stages were determined by postoperative pathological, clinical, and/or radiographical data.

The clinicopathological characteristics of the patient groups are summarized in Supplemental Tables S1 and S2 available at *http://ajp.amjpathol.org*. The study endpoint examined for lung and kidney cancers was disease-specific death. The survival time, in months, was the period from disease diagnosis, or from surgery, to death (lung and kidney, respectively). Patients alive at last follow-up or those with deaths not attributable to disease were censored at last follow-up. Death of unknown cause was censored for lung cancers; all causes were known for kidney cancer patients. The endpoint for prostate cancers was disease recurrence, defined as a postoperative serum prostate-specific antigen of 0.2 ng/ml or greater. Patients without recurrence were censored at last follow-up. The Eastern Cooperative Oncology Group performance status was determined at initial presentation for kidney and lung cancers.

Lung Cancer Patients

The World Health Organization histological classifications of carcinomas of the lung were used. The lung cancer TMA contained 285 patient samples of which 262 (92%) were clinically informative. Of 262 cases 257 (98%) were also informative for H3K18ac and H3K4me2. Adenocarcinomas included tumors with bronchioloalveolar components. The lung tumors were graded according to the American Joint Committee on Cancer Cancer Staging Manual. The median age of lung cancer patients in this cohort was 67 years (range, 41 to 87 years) and the male to female ratio was 1:1.4. The median tumor size was 2.5 cm. The median follow-up in this cohort was 59.0 months (range, 1.0 to 229 months).

Kidney Cancer Patients

Pathological tumor subtyping of kidney cancers was performed according to the 1997 International Union Against Cancer/American Joint Committee on Cancer classification of malignant tumors. Kidney tumors were taken from radical or partial nephrectomies of patients with renal cell carcinoma. Of the 379 cases on the TMA, 373 (98%) were clinically informative with a further 359 (96%) being informative for H3K18ac, H3K4me2, and H3K9me2. The median age of kidney cancer patients in the localized cohort was 63.5 years (range, 27 to 88 years) and the male to female ratio was 1.9:1. The median tumor size was 4.5 cm. The median follow-up in this cohort was 43.1 months (range, 0.0 to 142 months).

Prostate Cancer Patients

Prostate cancers were all of the histological type adenocarcinoma, conventional, not otherwise specified. From 226 prostate cancer patients on the TMA who underwent radical retropubic prostatectomy, 212 were clinically informative, of which 185 (87%) were also informative for H3K9me2. Prostate grading was performed using the Gleason score system (equivalent to Gleason sum); low grade in our cohort included those cases of Gleason score 2 to 6. The median age of prostate cancer patients in this cohort was 64 years (range, 46 to 75 years). The median follow-up in this cohort was 60.0 months (range, 2.0 to 120 months).

IHC and Western Blotting

A standard two-step indirect IHC staining method was used for all antibodies as previously described[16] using the DAKO Envision system (DAKO, Carpinteria, CA). Primary rabbit anti-histone polyclonal antibodies were applied for 60 minutes at room temperature—for lung TMAs, H3K18ac[17] at 1:300 and H3K4me2 (Upstate, Lake Placid, NY) at 1:600 dilutions; for kidney TMA, H3K18ac at 1:400 and H3K9me2 (Abcam, Cambridge, MA) at 1:50 and H3K4me2 at 1:800 dilutions; for prostate TMA, H3K9me2 at 1:100; and for cell line IHC, H3K9me2 at 1:100 dilution from stock. The polyclonal rabbit anti-H3 (Abcam) was used at 5 μg/ml. Monoclonal anti-Ki-67 MIB-1 (7.5 μg/ml) and anti-human p53 DO-7 (15 μg/ml, DAKO) were used for Ki-67 and p53 detection, respectively. Using a test TMA containing 20 to 40 cases, we optimized the concentration of each antibody to observe the greatest variation in the staining range within each tissue type. The sections were counterstained with Harris' hematoxylin. Negative controls were identical array sections stained minus the primary antibody. For Western analysis, histones were acid-extracted from PC3 (bone metastasis of prostate cancer; American Type Culture Collection, Manassas, VA) and LNCaP (lymph node metastasis of prostate cancer; American Type Culture Collection) cell lines and subjected to standard Western blotting.

Scoring of IHC for All Tissues

Semiquantitative assessment of antibody staining on the TMAs was performed by pathologists blinded to all clinicopathological variables. Two pathologists scored all of the TMAs but one per cancer set (lung TMA-V.M., kidney and prostate TMAs-H.Y.). We chose IHC and semiquantitative analysis to generate the datasets because this is by and large the most common immunostaining method in clinical pathology settings, making our approach easily adoptable into current pathology laboratories. Only cancerous epithelial tissues were scored, and only primary tumor cells from the first surgery was included in the study. The lower acceptable limit for scoring a given tissue spot was 10 cells. However, in the majority of tumor spots there were between 100 and 1000 cells, and for most cases the tumor was represented by more than one spot containing the target tissue (average marker-informative primary tumor tissue spots per case = 3.1 for kidney, 2.4 for lung, and 3.0 for prostate). Normal epithelium in cancer specimens, mesenchymal or infiltrating inflammatory cells, and metastases were excluded from scoring. The frequency of positive nuclear expression (range, 0 to 100%) was scored for each TMA spot using the labeling index method. To produce a single representative staining for each case, the percent cell positivity from each tumor spot within each case was pooled and used to determine the percentile rank of patients in each dataset.

Statistical Analysis

To test whether ordinal variables differed across groups, we used the Kruskal-Wallis test, a nonparametric multigroup comparison test. To visualize the survival distributions, we used Kaplan-Meier plots. A multivariate Cox proportional hazards model was used to test the statistical independence and significance of multiple predictors. The proportional hazard assumption was tested using scaled Schoenfeld residuals. To study whether the categorized histone expression groupings differed across patient strata, we used the Fisher's exact test. Log-rank tests were used to test the difference between

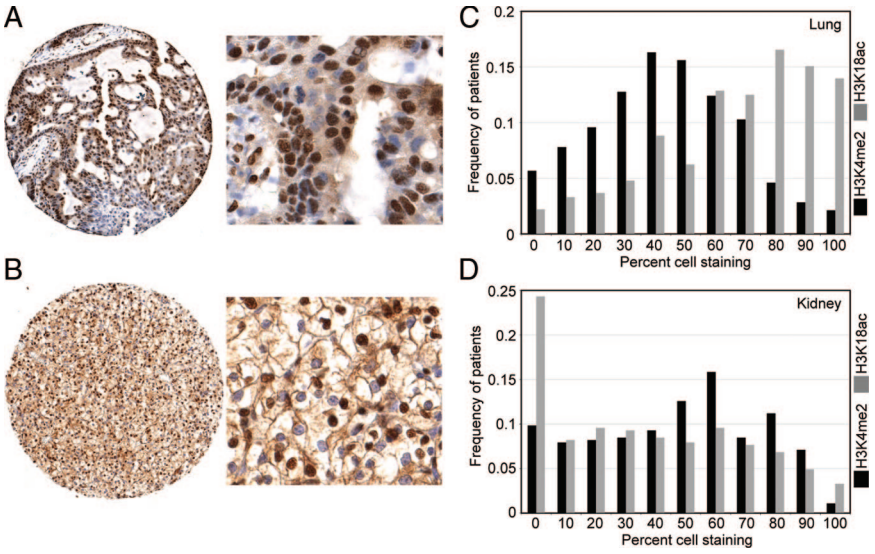

Figure 1. Cellular heterogeneity in levels of histone modifications in primary cancer tissues. Immunohistochemical staining of cancer tissues from lung adenocarcinoma (grade 2) (**A**) and kidney clear cell carcinoma (grade 1) (**B**) with an anti-H3K18ac antibody. Percentage of cancer cells with brown nuclei determines the global levels of each histone modification for a given individual. Distribution of patients for the levels of H3K4me2 (black bars) and H3K18ac (gray bars) in cancer tissues from lung (**C**) and kidney (**D**) are shown. The graphs represent the fraction of patients (*y* axis) with indicated levels of histone modifications as percent cell staining (*x* axis). Original magnifications: ×10 (**A**, **B**; **left**); ×40 (**A**, **B**; **right**).

survival distributions. A P value <0.05 was considered significant.

Chromatin Immunoprecipitation (ChIP) and Microarray Hybridization

ChIP was performed essentially as described.[18] Briefly, formaldehyde was added for 10 minutes at 37°C to growing cultures of cells. After PBS washing, cross-linked cells were scraped from the plates and washed with 1 ml of PBS containing protease inhibitors (Roche, Indianapolis, IN). Cells were lysed, incubated for 10 minutes on ice, and immediately sonicated. One hundred μl of the lysate were used for immunoprecipitation with anti-H3K9me2 or H3K18ac antibody; 10 μl of the lysate were used as input. After overnight reversal of crosslinking at 65°C, ChIPed and input samples were treated with RNase A for 30 minutes at 37°C and subsequently purified using the Qiagen (Valencia, CA) Qiaquick PCR purification kit. Ten ng of each IP and INP DNA were amplified using the WGA Kit (Sigma, St. Louis, MO). Two μg of amplified material were labeled with Cy3 or Cy5 (Perkin Elmer, Emeryville, CA) using the Bioprime labeling kit (Invitrogen, Carlsbad, CA). DNA was mixed with 35 μl of random priming solution (Invitrogen Bioprime kit) to a final volume of 75 μl, boiled for 5 minutes, and quickly cooled in an ice-water bath for 5 minutes. The labeling reaction was completed with 60 U Klenow, dNTPs (0.12 mmol/L dATP, dGTP, and dTTP and 0.06 mmol/L dCTP), 1.28 mmol/L Cy3 and Cy5 for input, and ChIPed DNA, respectively, and incubated for 3 hours at 37°C. The labeled DNA was purified using Qiagen Qiaquick PCR purification kit and the incorporation was measured with Nanodrop (NanoDrop, Wilmington, DE). Hybridization onto the human promoter array (G4489A; Agilent, Santa Clara, CA), washing, and scanning were performed according to the manufacturer's instructions. The arrays were scanned using an Agilent DNA microarray scanner. Data extraction and analyses were performed using the Agilent Feature Extraction software (version 9.1.3.1) and Chip Analytics software (version 1.2). Probe signals were normalized with Lowess normalization.

Results

Detection of Cellular Histone Modifications by Immunostaining of Cancer Tissues

To determine the cellular levels of histone modifications in tissues obtained from patients, we combined IHC, a method for detecting the presence of specific antigens in cells, with TMAs,[16,19] for high throughput analysis of a large number of tissue samples.[20] We analyzed the levels of H3K4me2, H3K9me2, and H3K18ac, using antibodies that recognize these specifically modified residues,[7,17] on TMAs of lung, kidney, and prostate cancers. The choice of these cancers and the number of patients in each array were based on specimen availability with complete follow-up clinical data. Here, the global level of histone modifications refers to the percentage of cancer cells within each tissue sample that stained positively for a given antibody. This scoring system is used routinely and extensively for a wide range of biomarkers that are currently in clinical use in pathology laboratories. Shown in Figure 1, A and B, is representative cancer tissues from lung (Figure 1A) and kidney (Figure 1B) stained with anti-H3K18ac antibody (objective: ×10 left panel; ×40 right panel). The cells with brown nuclei are considered positively stained, and their percentage within the tumor tissue is determined. The lack of staining by the histone modification antibodies is unlikely attributable to inaccessibility of their respective antigen because an anti-H3 antibody, which recognizes histone H3 irrespective of modifications, stains positively in essentially all cells (see Supplemental Figure S1 available at *http://ajp.amjpathol.org*). The unstained cells may still contain the modifications at certain genomic loci but their levels are below the detection limits of IHC, signifying that bulk histone modifications are considerably decreased in these cells.

Grouping of Patients Based on Histone Modification Levels

To determine whether histone modifications predict clinical outcome, we first stratified patients into broad categories based on clinicohistological features such as grade or stage. The rationale for this initial stratification is that grade and stage are strong predictors of outcome.[8] Grade is a histological measure of tumor differentiation. Stage is a measure of tumor size and spread beyond its original site. In general, higher grade and stage are associated with poorer outcome. However, within cancers that are of equivalent grade and stage, there are subtypes of patients that are molecularly heterogeneous and have different clinical outcomes.[8] Prognostic biomarkers are therefore needed to subclassify patients beyond grade and stage into more clinically cohesive groups. After grade or stage stratification, we assigned patients from each category into two groups according to a specific histone modification pattern or histone pattern for short. This histone pattern was derived initially from an unsupervised clustering of prostate cancer patients, based on the cellular levels of H3K4me2 and H3K18ac staining that predicted clinical outcome. We did not search for new cut-off values for these two modifications in the current study. The histone pattern predicts that the patients with lower levels of H3K4me2 and H3K18ac have poorer prognosis than those with higher levels. After application of the histone pattern to patients in each cancer of lung and kidney, we tested the prediction that the two resulting groups should have significantly different clinical outcomes.

Histone Modifications Predict Survival Probability in Lung Cancer

To assess the distribution of staining for H3K4me2 and H3K18ac, we plotted the frequencies (*y* axis) of tissue samples in which the indicated percentage cell staining (*x* axis) were observed for each modification (Figure 1C). H3K4me2 staining showed a broad distribution whereas H3K18ac staining was skewed toward higher percent cell staining (Figure 1C). To determine whether histone modification patterns are clinically informative in lung cancer, we first partitioned the patients into stages 1 through 4 (see Supplemental Figure S2 available at *http://ajp.amjpathol.org*). Within each stage, the patients were then assigned to two groups according to the predictive histone modification pattern that we identified from prostate cancer. The tumors with high levels of H3K4me2 and H3K18ac were assigned to group 1 (ie, H3K4me2 >60 or H3K4me2 and H3K18ac >35 percentile staining); the remaining tumors with lower levels of the modifications were assigned to group 2. In stage 1 lung adenocarcinoma ($n = 159$), we found that the patients in group 2 with lower cellular levels of histone modifications (red line, Figure 2A) had a significantly lower 15-year survival probability compared with those in group 1 (black line, Figure 2A) (log rank, $P = 0.018$, hazard ratio (HR) = 2.19, 95% CI = 1.13 to 4.27). Between the two groups, there was no difference in gender or age at surgery, but there was a statistically significant difference in grade distribution ($P = 0.0026$). Paradoxically, the difference in grade distribution was attributable to presence of more low-grade tumors in group 2 patients with poorer outcome (Figure 2A, inset box; and see Supplemental Table S1 available at *http://ajp.amjpathol.org*). In stages 2 ($n = 42$), 3 ($n = 40$), and 4 ($n = 16$), we did not detect subgroups with significant differences in clinical outcome. Thus, the same prognostic histone modification pattern in prostate cancer serves as marker of prognosis in stage 1 lung adenocarcinoma.

The Histone Pattern Is an Independent Prognosticator in Lung Cancer

To determine how the histone modifications compare with other known biomarkers in lung cancer, we examined the percentage of cells that stain positively for p53, which, when overexpressed, is associated significantly with poor patient outcome in stage 1 adenocarcinoma.[21] The expression levels of p53 were different in the two histone groups, with lower expression in the group with the poorer prognosis, 32.1% average positivity in group 1 and 19.7% in group 2 ($P = 0.033$). So, the poorer prognosis predicted by the histone pattern is not attributable to increased incidence of p53 mutation. Additionally, in groups 1 and 2, 30 and 25% of patients had a mitotic count >0, respectively ($P = 0.64$), suggesting that the prognostication by the histone pattern is not attributable to increased proliferation rate. Finally, in a multivariate Cox model that included grade, mitotic count, p53, patients' performance status (Eastern Cooperative Oncology Group), the histone groupings remained a significant predictor of outcome (Table 1). Thus, the histone modification patterns are independent predictors of clinical outcome in lung adenocarcinoma.

Histone Modifications Predict Survival Probability in Kidney Cancer

In kidney carcinoma, there was a broad distribution of staining levels for both H3K4me2 and H3K18ac with <10% of specimens showing 90 to 100% staining (Figure 1D). Applying a similar histone pattern as above (ie, >60 or >35 percentile staining for H3K4me2 and H3K18ac, respectively) to the patients with localized kidney tumors ($n = 192$; see Supplemental Figure S3 available at *http://ajp.amjpathol.org*), we identified two groups of patients that differ significantly in their survival probabilities (Figure 2B). The patients with low levels of both modifications (group 2) had a significantly poorer 1-year survival probability than those with higher levels of histone modifications (group 1) (log rank, $P = 0.028$, HR = 2.22, 95% CI = 1.07 to 4.62). There was no difference in the distribution of patients in the two groups according to gender, age at surgery, grade, or stage (Figure 2B, inset box; and see Supplemental Table S1 available at *http://ajp.amjpathol.org*). In patients with metastatic disease ($n = 163$), we did not detect subgroups with distinct clinical outcomes (see Supplemental Figure S4A available at *http://ajp.amjpathol.org*).

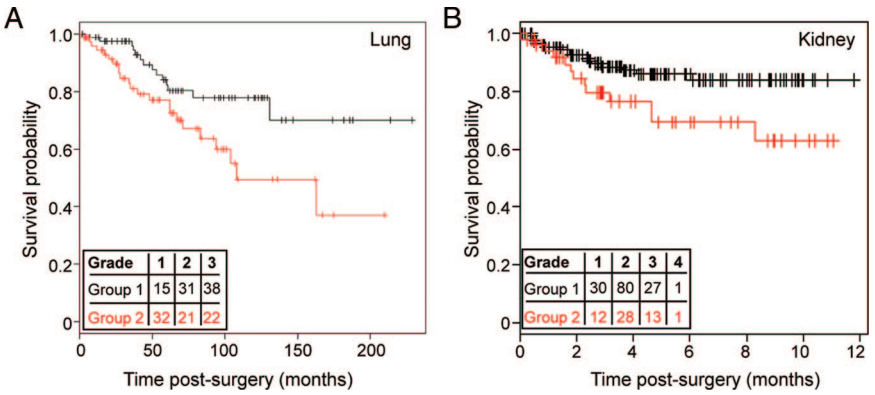

Figure 2. Prediction of clinical outcome in different carcinomas by histone modifications. For each cancer type, patients were first assigned to two groups based on the levels of H3K4me2 and H3K18ac, and then their clinical outcomes were compared. Kaplan-Meier plots are used to visualize survival probabilities of the two groups (group 1, black line; group2, red line) in lung (**A**) (log rank, $P = 0.018$, $n = 159$) and kidney (**B**) (log rank, $P = 0.028$, $n = 192$). Tabulated in the **insets** is the distribution of the patients in each group according to grade.

When patients were stratified only based on grade, the histone pattern distinguished two groups with significantly different survival probabilities in grades 1 and 2 but not in grades 3 and 4 cancer (see Supplemental Figure S4B available at *http://ajp.amjpathol.org*). Thus, as in prostate and lung cancers, lower levels of the same two histone modifications predict poor clinical outcome in localized kidney adenocarcinoma.

The Histone Pattern Is an Independent Prognosticator in Kidney Cancer

To determine how the histone modifications compare with other known biomarkers in kidney cancer, we examined the percentage of cells that stain positively for Ki-67, a marker of proliferation, and p53. Increased expression of Ki-67 or p53 was shown previously to be associated significantly with poor patient outcome in kidney adenocarcinoma.[22,23] The median Ki-67 expression levels were essentially the same in the two histone groups, 5% in group 1 and 5% in group 2 ($P = 0.50$), indicating that the histone groupings are not attributable to their proliferation status. The expression levels of p53 were different in the two histone groups, with lower mean expression in the group with the poorer prognosis, 7.3% in group 1 and 3.2% in group 2 ($P = 0.0002$). So, the poorer prognosis predicted by the histone modifications is not attributable to increased incidence of p53 mutation. In a multivariate Cox model that included grade, Ki-67, and p53, the histone grouping remained a significant predictor of outcome (Table 1) but not when Eastern Cooperative Oncology Group performance status was also included. Thus, the histone modification patterns are predictors of outcome in localized kidney cancer independently of grade, proliferation rate, and p53 expression.

Cellular Levels of H3K9me2 Predict Clinical Outcome in Prostate and Kidney Cancers

Both H3K4me2 and H3K18ac are modifications associated with gene activity. We next asked whether lower

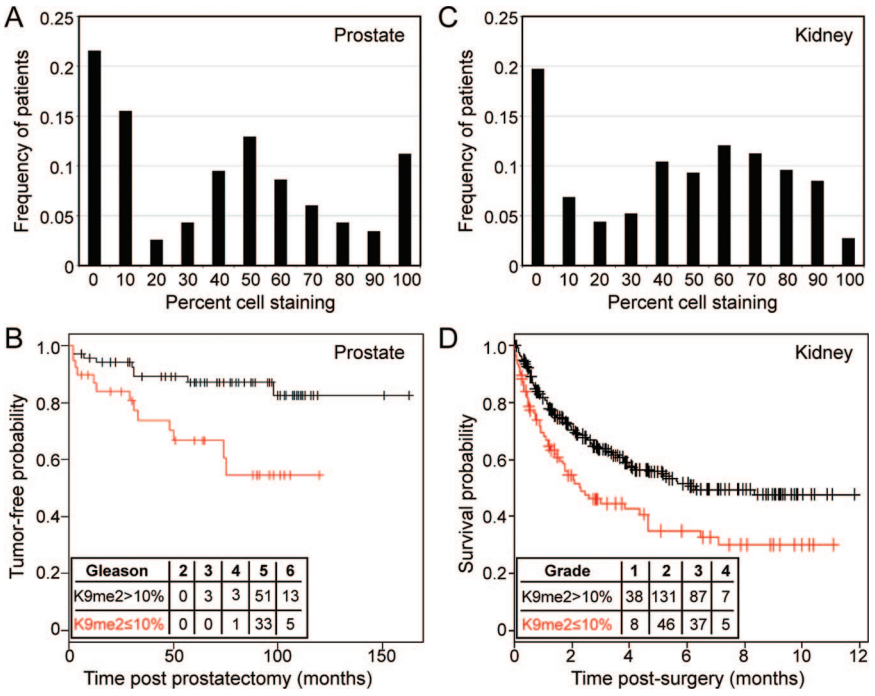

Figure 3. The cellular levels of H3K9me2 predict clinical outcome in prostate and kidney cancers. Distribution of patients for the levels of H3K9me2 in cancer tissues from prostate (**A**) and kidney (**C**) are shown. The graphs represent the fraction of patients (y axis) with indicated levels of histone modifications as percent cell staining (x axis). For each cancer type, patients were first assigned to two groups based on the levels of H3K9me2, and then their clinical outcomes were compared (group 1, H3K9me2 >10%, black line; group 2, H3K9me2 ≤10%, red line). Kaplan-Meier plots are used to visualize the difference in outcome of the two groups in low-grade prostate (**B**) (log rank, $P = 0.0043$, $n = 109$) and all kidney (**D**) (log rank, $P = 0.00092$, $n = 359$) cancer patients. Tabulated in the **insets** is the distribution of the patients in each group according to grade.

Table 1. Multivariate Proportional Hazard Analyses

Variable	Hazard ratio	95% CI	*P* value
Histone pattern in stage 1 lung cancer (H3K4me2 and H3K18ac)			
Histone pattern grouping	4.94	1.67 to 14.63	4.0E-3
Grade	1.35	0.60 to 3.02	4.7E-1
Mitotic count	1.62	0.49 to 5.35	4.2E-1
ECOG performance status	2.00	0.74 to 5.42	1.7E-1
p53 levels	1.01	0.99 to 1.02	4.0E-1
Histone pattern in localized kidney cancer (H3K4me2 and H3K18ac)			
Histone pattern grouping	2.29	1.01 to 5.21	3.4E-2
Grade	2.20	1.25 to 3.88	6.4E-3
Ki67 levels	1.01	0.97 to 1.06	6.5E-1
p53 levels	1.03	1.01 to 1.05	1.6E-2
H3K9me2 in low-grade prostate cancer			
H3K9me2 grouping	2.95	1.08 to 8.00	3.4E-2
Preoperative serum PSA (ng/ml)	1.05	1.01 to 1.08	6.8E-3
Grade	1.21	0.37 to 3.92	7.5E-1
Stage	2.85	0.97 to 8.42	5.8E-2
Capsule invasion	2.68	1.12 to 6.40	2.7E-2
H3K9me2 in all kidney cancer			
H3K9me2 grouping	1.85	1.31 to 2.62	5.4E-4
Tumor localization	0.14	0.09 to 0.22	<E-12
Grade	1.39	1.07 to 1.79	1.2E-2
Ki67 levels	1.01	1.00 to 1.03	1.6E-1
p53 levels	1.02	1.01 to 1.03	7.8E-5
H3K9me2 in localized kidney cancer			
H3K9me2 grouping	2.26	1.03 to 4.92	4.1E-2
Grade	2.12	1.17 to 3.85	1.3E-2
Ki67 levels	1.01	0.97 to 1.06	6.2E-1
p53 levels	1.02	1.00 to 1.04	3.9E-1

levels of H3K9me2—a modification associated with both gene repression and activity as well as heterochromatin—also predicts poorer prognosis in cancer. We determined H3K9me2 cellular levels in the same prostate and kidney cancer TMAs in which other modifications were examined. Distribution of staining in both prostate and kidney cancer specimens showed a broad pattern, ranging from 0 to 100% staining (Figure 3, A and C). In prostate cancer, cellular levels of H3K9me2 were not predictive of outcome among patients with high Gleason score tumors (score ≥ 7, $n = 76$). However, among the low Gleason score tumors (score <7, $n = 109$), the levels of H3K9me2 as a continuous, undichotomized variable was significantly related to tumor recurrence (Cox regression, $P = 0.0037$). Using Rpart tree, we then determined an optimal cut point in the levels of H3K9me2 to dichotomize patients into high and low levels of H3K9me2. As shown in Figure 3B, patients with $\leq 10\%$ H3K9me2 staining (group 2; red line) showed a higher risk of tumor recurrence compared with patients with $>10\%$ staining (Cox proportional hazard, $P = 0.0043$, HR = 3.25, 95% CI 1.38 to 7.63). The prognostication by H3K9me2 was independent of tumor grade (Figure 3B, inset), stage, preoperative prostate-specific antigen, and capsule invasion within the low Gleason score group (Table 1).

We next determined whether lower levels of H3K9me2 also predicts poorer prognosis in kidney cancer patients. Indeed the levels of H3K9me2 as a continuous, undichotomized variable was significantly related to survival probability in all kidney cancer patients (Cox regression, $P = 0.028$, $n = 359$) and in patients with localized cancer (Cox regression, $P = 0.026$, $n = 189$). Using the same cut point as in prostate cancer, kidney cancer patients with $\leq 10\%$ H3K9me2 staining (group 2, red line) showed significantly decreased survival probability compared with patients with $>10\%$ staining (Cox proportional hazard, $P = 0.00092$, HR = 1.7, 95% CI 1.3 to 2.4; Figure 3D). This was true for all patients and also within localized or metastatic disease strata (see Supplemental Figure S5 available at *http://ajp.amjpathol.org*). In a multivariate Cox model that included grade, Ki-67, p53, and/or tumor localization, levels of H3K9me2 remained a significant predictor of outcome (Table 1). Taken together, our data indicate that lower cellular levels of H3K9me2 also predict poor prognosis in prostate and kidney cancers.

Changes in Global Levels of Histone Modifications Correlate with Their Levels at Repetitive DNA Elements

To determine how cellular patterns of histone modifications map to individual promoters at the molecular level, we identified two prostate cancer cell lines that may serve as a model for observations in primary tumors. We expected the phenotypically more aggressive cancer cell line to contain generally lower levels of histone modifications. This was indeed the case for the LNCaP and PC3 prostate cancer cell lines. The PC3 cell line, derived from a bone metastasis of prostate cancer, is considered to be more aggressive than the LNCaP line, which was isolated from a lymph node metastasis. Figure 4A shows immu-

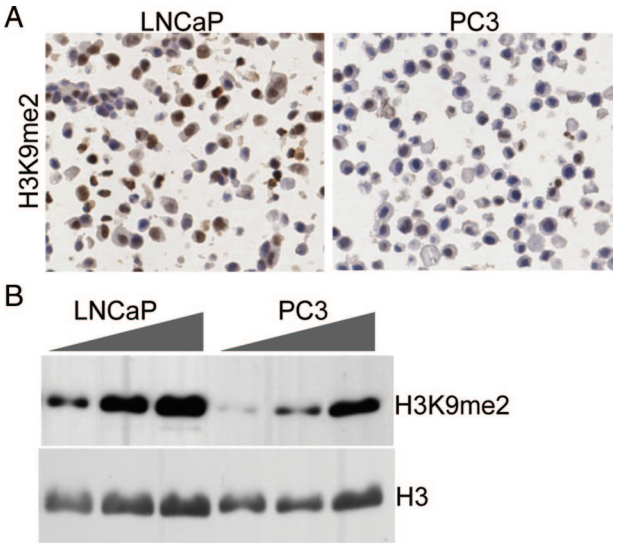

Figure 4. Cellular heterogeneity in levels of histone modifications in cancer cell lines. **A:** Immunohistochemical examination of H3K9me2 in LNCaP and PC3 prostate cancer cell lines. Note the increased percentage of PC3 cells with lower levels of H3K9me2 (blue nuclei) compared with LNCaP cells. **B:** Western blot of acid-extracted histones from LNCaP and PC3 cells for H3K9me2 levels and histone H3 (irrespective of modifications) as a loading control. The triangles indicate increased loading from left to right.

nohistochemical staining of LNCaP and PC3 cells with an anti-H3K9me2 antibody. The more aggressive PC3 cells contained reduced H3K9me2 levels compared with LNCaP cells. Western blotting of acid-extracted histones confirmed the IHC results (Figure 4B). PC3 cells also showed lower levels of H3K18ac and H3K4me2 compared with LNCaP cells (see Supplemental Figure S6 available at *http://ajp.amjpathol.org*).

We next performed ChIP-chip (chromatin immunoprecipitation combined with microarrays) experiments to compare the H3K9me2 distribution between LNCaP and PC3 cells at promoters genome-wide (Figure 5A). For each cell line, we compared the ChIPed DNA with an anti-H3K9me2 antibody to total genomic DNA (input). We used an Agilent human promoter array containing 17,054 promoters, covering an average region from −5.5 kb to +2.5 kb with respect to the annotated transcription start site of each promoter. The data for each gene was standardized to generate 16 500-bp fragments represented as columns in Figure 5A. We found that distribution of H3K9me2 in LNCaP and PC3 cells were very similar with a high degree of correlation at each position across the promoters genome-wide (Figure 5B). So, the difference in total levels of H3K9me2 between LNCaP and PC3 cells is likely not attributable to global changes at gene promoters.

We next asked whether lower global levels of histone modifications in PC3 cells were attributable to decreased levels at repetitive DNA elements. These DNA elements, which collectively comprise ~70% of the human genome, are significantly DNA demethylated and have lower levels of H4K16ac and H4K20me3 in certain cancers.[6] We used the same ChIPed DNA as above followed by quantitative real-time polymerase chain reaction (qRT-PCR) to examine the levels of H3K9me2 at several DNA repetitive elements (Figure 5C). To circumvent copy number variation, for each repetitive DNA element, we examined the

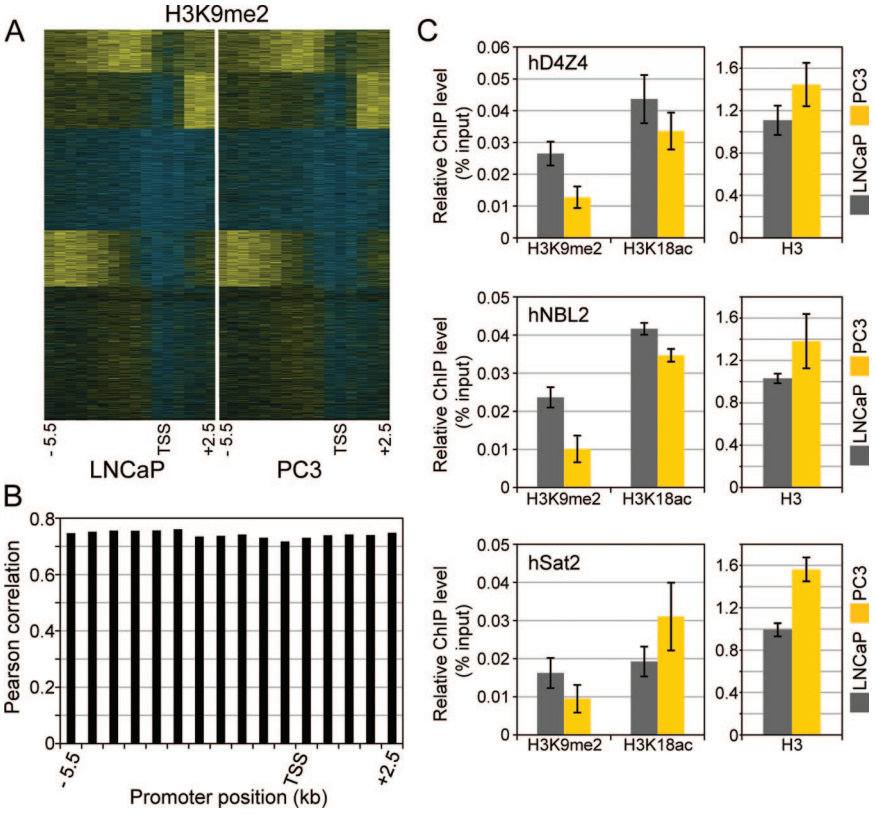

Figure 5. Global levels of H3K9me2 correlates with its levels at repetitive DNA elements. **A:** ChIP-chip analysis of H3K9me2 in LNCaP and PC3 cells. Each row represents the region from −5.5 to +2.5 of annotated transcription start site (TSS) for a given gene that is divided into 16 fragments of 500 bp each. Genes are grouped based on similarity of e1a-binding pattern across the 8-kb promoter region. The colors indicate relative enrichment or depletion of ChIPed DNA (yellow) versus input (blue) from each cell. **B:** Correlations of H3K9me2 levels at each of the 16 fragments across all promoters between LNCaP and PC3 cells. **C:** ChIP quantitative real-time PCR analyses of the levels of H3K9me2 and H3K18ac at the indicated DNA repetitive elements. The values are represented as percentage of input. The error bars represent SD of three independent experiments. Histone H3 ChIP was used as a control to show that lower modification levels in PC3 cells are not attributable to nucleosome loss.

region at the boundary of repetitive and nonrepetitive DNA elements. As shown in Figure 5C, PC3 cells showed lower levels of H3K9me2 at subtelomeric repeat elements (D4Z4), a tandem 1.4-kb element found in acrocentric chromosomes (NBL2) and juxtacentromeric satellite 2 (Sat2) DNA sequences. Lower H3K9me2 levels were not attributable to histone loss (Figure 5C). H3K18ac also showed lower levels at D4Z4 and NBL2 elements. These results indicate that global loss of histone modifications in more aggressive cancers correlate with lower levels of the modifications at DNA repetitive elements.

Discussion

We have provided evidence that the global levels of the same histone modifications in cancer tissues predict disease outcome in different adenocarcinomas of lung and kidney in addition to the previously reported prostate cancer.[7] Generally in each cancer, patients who have a lower percentage of cancer cells that stain positively for H3K4me2 and H3K18ac have poorer prognosis than those with higher percentages. Interestingly, the cellular level of H3K9me2 is also associated with disease outcome, with lower levels predicting poorer prognosis in prostate and kidney cancers (we have not yet examined H3K9me2 in the lung cancer cohort). Thus, the general picture that emerges from our data are that the lower cellular levels of histone modifications are associated with poorer clinical outcome. Interestingly, the levels of histone modifications are correlated positively with each other, suggesting that loss of one histone modification is generally associated with loss of other modifications within a patient (see Supplemental Figure S7 available at *http://ajp.amjpathol.org*). Other laboratories have validated and extended the prognostic powers of histone modifications to yet other modifications and other cancers including non-small cell lung cancer[24] and cancers of breast, ovary, and pancreas.[25] This general applicability of histone modification patterns is unlike most prognostic markers described today. The prognostic power of the histone modifications is independent of clinicopathological variables including proliferation rate as well as certain biomarkers such as p53 expression in lung and p53 and Ki-67 expression in kidney cancers. Therefore, the cellular patterns of histone modifications add further nonredundant information to the current prognostic markers for prediction of clinical behavior in cancer patients.

Analyses of histone modifications in cancer have typically focused on specific genomic loci such as individual gene promoters, revealing local perturbation of histone modifications with consequent effects on the expression of downstream genes. Extending this notion to the PC3 cells, which contain ~50% less H3K9me2 compared with LNCaPs, we were surprised to find that ChIP-chip data from the two cell lines were essentially similar to each other. This suggests that differences in global levels of histone modifications are unlikely to arise from changes at gene promoters. However, ChIP analyses of three DNA repetitive elements showed decreased H3K9me2 levels in PC3 versus LNCaP cells. Such correlations between global levels of histone modifications and their levels at repetitive elements, but not at gene promoters, were demonstrated previously for other cancers.[6] Because DNA repeat elements comprise ~60 to 70% of genomic sequences,[26] levels of histone modifications at these regions may account for the global differences observed in both cancer cell lines as well as in primary cancer tissues.

The repetitive elements are demethylated on DNA in cancer, which may contribute to genomic instability.[2] Our data and those of others[6] now suggest that the repetitive elements may also get demethylated and/or deacetylated on their associated histones. The biological consequence of this demodification of histones at repetitive elements is unclear but is likely associated with a more aggressive phenotype because lower global levels of histone modifications predict poorer prognosis. The regulatory mechanisms that affect histone modifications at the repetitive elements are poorly understood but could be attributable to improper targeting, altered expression, and/or activity of histone-modifying enzymes through genetic mutations, expression changes, and/or posttranslational control.[27] Because all histone modifications are reversible, increased activity of one set of histone modifiers, eg, HDACs, could change the overall states of histone modifications to cause detectable changes at a global level.[28] Some of these histone modifiers may preferentially affect DNA repetitive elements. Although this has not been demonstrated for mammalian proteins, the Hos3 HDAC in yeast preferentially deacetylates the ribosomal DNA repeats.[29]

In potentially related studies, we have shown that viral oncoproteins, such as the adenovirus e1a, can alter global patterns of histone modifications in human cells through genome-wide redistribution of specific histone modifiers away from most of the genome and restricting them to a limited but biologically related set of genes to favor cell replication and thus viral production.[30,31] As in the case of the e1a oncoprotein, loss of histone modifications at the DNA repetitive elements in primary cancers could also reflect redistribution of histone acetyl- and methyltransferases away from these regions and onto a smaller set of genes that confer an advantage to the cells in which this occurs. Whatever the mechanism, it remains to be determined whether the cells with little or no detectable histone modifications are derived from a single precursor cell (ie, clonal) or from parallel loss of histone modifications in different tumor cells within a tissue.

The prognostication by the histone modifications might have implications for epigenetic therapy. One possibility is that the patients with poorer outcome who have low levels of H3K4me2, H3K18ac, and/or H3K9me2 would benefit more from HDAC inhibitors than those with high levels of the histone modifications. It is also possible that the poor outcome group would require a different regimen of various epigenetic therapeutics.[32,33] Whatever the case may be, the simplicity and robustness of our approach should facilitate the development of a standard and effective epigenetic assay to identify subsets of cancer patients with similar clinical outcome.

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